Degradation of Peroxisomes after Transfer of Methanol-Grown Hansenula polymorpha into Glucose-Containing Media

Veenhuis, M.; Zwart, K.; Harder, W.

Published in:
FEMS Microbiology Letters

DOI:
10.1016/0378-1097(78)90007-1

FEMS Microbiology Letters 3 (1978) 21–28

Published by Elsevier/North-Holland Biomedical Press

DEGRADATION OF PEROXISOMES AFTER TRANSFER OF METHANOL-GROWN *HANSENULA POLYMORPHA* INTO GLUCOSE-CONTAINING MEDIA

M. VEENHUIS, K. ZWART and W. HARDER

Laboratory of Electron Microscopy and Department of Microbiology, Biological Centre, University of Groningen, Kerklaan 30, Haren, The Netherlands

Received 1 November 1977

1. Introduction

It is now well established that yeasts growing on methanol as the sole source of carbon characteristically contain peroxisomes [1–4], whose number and ultrastructure, typified by a more or less extensive crystalline matrix, is dependent upon growth conditions. Biochemical and cytochemical studies have revealed that these organelles contain high levels of alcohol and catalase [5–9], which are known to be the key enzymes of methanol oxidation in these organisms [10]. During growth on glucose, peroxisomes – and the enzymes associated with them – are virtually absent from the yeast cells and the study of the development and disappearance of these organelles is therefore of considerable interest.

Biochemical experiments [11] have indicated that in *Kloeckera* sp. 2201 the synthesis of catalase is inhibited after the transfer of methanol-grown cells into media containing glucose. A similar repression of enzymes involved in methanol oxidation by glucose has been reported in *Candida boidinii* [12]. The kinetics of the decrease of specific activity of catalase in the culture of *Kloeckera* sp. 2201 after the transfer to glucose media indicated a dilution of enzyme molecules over newly formed cells. However, electron microscopic observations, which revealed the disappearance of peroxisomes within 8 h after the transfer, led to the suggestion that these organelles might have been degraded [11]. This poses the question whether degradation of peroxisomes may occur without simultaneous degradation of one of its characteristic enzymes [6].

The present communication describes the rapid decrease of alcohol oxidase and catalase activity in cells of *Hansenula polymorpha* following the addition of glucose to cultures growing on methanol. This loss of activity of both enzymes is associated with rapid degradation of peroxisomes.

2. Materials and Methods

2.1. Organism and cultivation

Hansenula polymorpha de Morais et Maya CBS 4732 was used in all experiments. The organism was grown at 37°C in 500 ml erlenmeyer flasks containing 100 ml basal medium supplemented with 0.5% (v/v) methanol as described previously [1]. Glucose was added to exponentially growing cultures or cultures which had been in the stationary phase for 12 h to give a final concentration of 0.4% (w/v).

2.2. Preparation of spheroplasts

Spheroplasts were prepared by treatment of suspensions of whole cells with "Zymolyase" [13] for 15 min at 37°C according to the procedure described by Osumi et al. [14].

2.3. Enzyme assays

Alcohol oxidase was determined with an oxygen electrode as described by van Dijken et al. [15]. The activity of alcohol oxidase is expressed as μmoles of oxygen consumed min^{-1} · mg $protein^{-1}$. Catalase was assayed by the spectrophotometric method of

Lück [16]. Catalase activity is expressed as ΔE_{240} · min^{-1} · mg $protein^{-1}$. Protein was determined by the method of Lowry et al. [17], using bovine serum albumin as a standard.

2.4. Cytochemical staining

Cytochemical staining procedures for the demonstration of catalase and alcohol oxidase activity were performed as described previously [6].

2.5. Freeze-etching

Cells were incubated for 15 min in 15% (v/v) glycerol, frozen in FREON and freeze fractured in a Balzers freeze-etch unit, according to the technique described by Moor [18].

2.6. Fixation and postfixation

Whole cells were fixed with 1.5% $KMnO_4$ for 20 min at room temperature. Spheroplasts were post-fixed for 45 min in a solution of 1% OsO_4 and 2½% $K_2Cr_2O_7$, in 0.1 M cacodylate buffer, pH 7.2. After dehydration in a graded alcohol series the cells were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

2.7. Morphometrical analysis of thin sections

The average number of peroxisomes was estimated by at random counting of cell profiles in thin sections. For each sample at least 500 cells were counted. Volume densities of peroxisomes in the cytoplasm were estimated with the point counting technique according to Weibel and Bolender [19]. Student's *t*-test was used for statistical analysis.

3. Results and Discussion

3.1 Biochemical observations

Addition of 0.4% glucose to a batch culture of methanol-grown *Hansenula polymorpha* which had been in the stationary phase for 12 h, resulted in growth without a lag (Fig. 1). Therefore, the enzymes

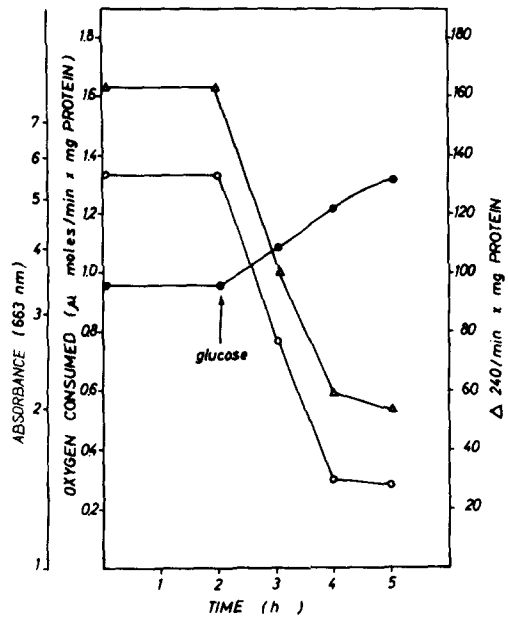


Fig. 1. Effect of addition of 0.4% glucose to a stationary methanol-grown batch culture of *Hansenula polymorpha*. ●——●, growth; ○——○, alcohol oxidase activity; △——△, catalase activity.

required for the metabolism of glucose must have been present in these cells. After the addition of glucose, a rapid decrease of alcohol oxidase activity was observed in the cells; in 2 h the activity measured in cell-free extracts had decreased to 36% of the initial value while in the same time interval the activity of catalase diminished to 36% of the initial activity.

When glucose (0.4%) was added to a culture of *Hansenula polymorpha* growing exponentially on methanol, a slight increase in growth rate was observed, associated with a loss of alcohol oxidase and catalase activity in the cells (Fig. 2). After incubation for 2 h, alcohol oxidase and catalase activities both had decreased to 20% of the initial values. These results differ from those of Yasuhara et al. [11] obtained with methanol-grown *Kloeckera* sp. 2201. After transfer of this organism into glucose media, the catalase activity continued to increase for 2 h while after this period the synthesis of this enzyme was completely repressed. However, no inactivation of catalase activity was reported. Our results with *Hansenula polymorpha* indicated an immediate inhibition of both catalase and alcohol oxidase synthesis after addition of glucose to the cell suspension, but

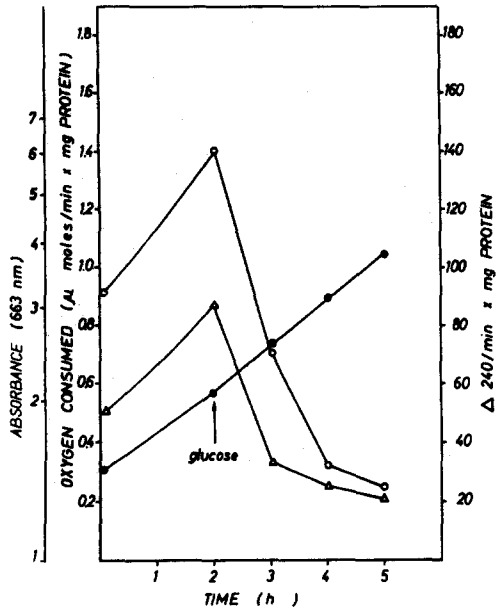


Fig. 2. Effect of addition of 0.4% glucose to an exponentially growing batch culture of *Hansenula polymorpha* on methanol. Symbols as in Fig. 1.

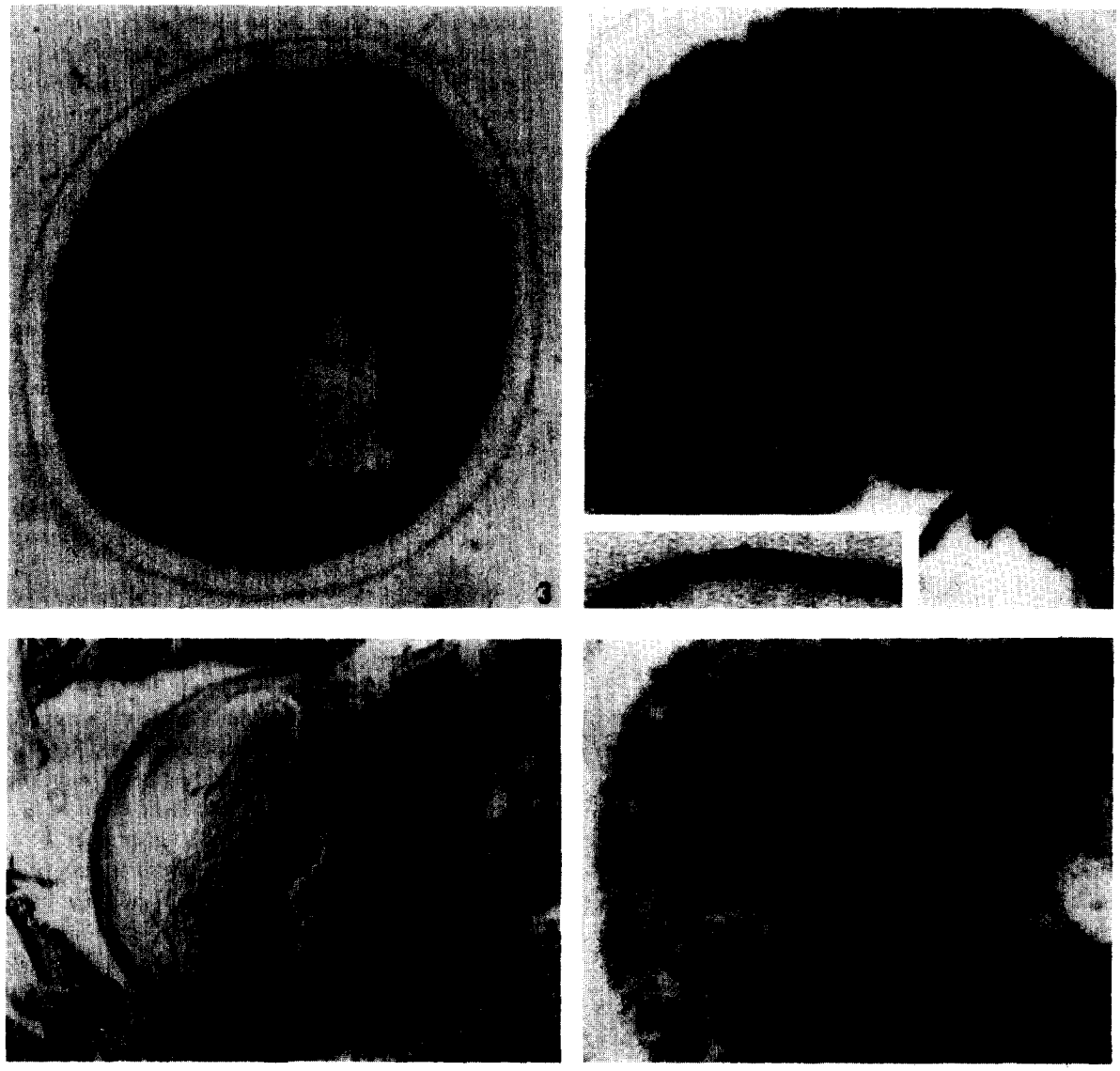
also suggested a rapid inactivation of already existing enzymes. This was indicated by the fact that the observed loss of enzyme activities could not be accounted for by dilution of enzymes – and thus peroxisomes – as a result of growth. Therefore the drastic decrease in both alcohol oxidase and catalase activity must be explained by a rapid inactivation – or possibly degradation – of these enzymes following the addition of glucose to the culture.

3.2. Electron microscopical observations

Sections of $KMnO_4$-fixed cells of *Hansenula polymorpha,* transferred from methanol media into a medium containing 0.4% glucose during the mid-exponential or stationary growth-phase, revealed that part of the peroxisomes in the cells was rapidly degraded. This phenomenon is illustrated in Figs. 3–12 in cells which were transferred during exponential growth on methanol. These cells generally contained one large peroxisome accompanied by one to four small adhering ones (Fig. 3). The process of degradation generally started on the large peroxisome – leaving the small ones apparently intact – and was initiated by the formation of several dark membraneous layers, which partly or completely surrounded the organelle (Fig. 4). Freeze-etch preparations showed that these membranes had a smooth appearance (Fig. 5) similar to the peroxisomal membrane [1] and glutaraldehyde –OsO_4 fixation indicated that at this stage the peroxisomes still contained a crystalline matrix. Subsequently this crystalline matrix disappeared and the dark layers were no longer observed (Fig. 6). The peroxisomes then were slightly swollen and showed a more or less round shape, while the membrane surrounding the organelles had changed from a structure of approximately 70 Å in width (Fig. 9) into a membrane of approximately 90 Å (Figs. 7, 9). Freeze-etch preparations revealed the occurrence of particles on the 90 Å membranes whereas the 70 Å membranes were smooth (Fig. 8), indicating that the surface structure of the membrane had also changed.

At this stage the first indications of disintegration became visible (Fig. 7) and membraneous invaginations and loose vesicles were observed in the peroxisomal matrix (Figs. 9, 10). As degradation of the peroxisome progressed, the areas of disintegration quickly increased in volume and in number (Fig. 9). Serial sections through disintegrating peroxisomes revealed that the process may be restricted to one large area or may occur in several smaller areas, which, in the latter case, generally were not in contact with each other but were found randomly distributed throughout the peroxisomes. The following stages of the disintegration (Figs. 11 and 12) indicate that the peroxisomal matrix was gradually converted into an amorphous structure with a fine granular appearance. Finally, after disintegration was completed, the peroxisome had turned into an organelle, which on the basis of morphological and ultrastructural features may be considered as a vacuole. For instance, in freeze-etch preparations, its surrounding membrane showed a structure similar to that of a typical yeast vacuolar membrane. Whether in any stage of peroxisomal degradation fusion of the organelle with an already existing vacuole had occurred, could not be established.

Although the general pattern of peroxisomal disintegration in exponential and stationary phase cells was comparable, two differences were observed. In stationary phase cells, which generally contained



Abbreviations in electron micrographs: L, lipoid droplet; n, nucleus; p, peroxisome; v, vacuole. Cells were fixed/post-fixed with $KMnO_4$ unless stated otherwise. The arrow in the freeze-etch micrographs represents the direction of shadowing.

Fig. 3. Thin section of a cell from the mid-exponential phase of a culture growing on methanol (37 000 ×).

Fig. 4. Detail showing membraneous layers around the peroxisome, 30 min after the addition of glucose (62 000 ×). A magnification (135 000 ×) is shown in the inset.

Fig. 5. Freeze-fractured cell showing smooth membraneous layers around the peroxisome (51 000 ×; compare Fig. 4).

Fig. 6. Change in morphology of the peroxisome due to disappearance of the crystalloid. Dark layers partly surround the organelle (50 000 ×).

5–7 large peroxisomes [1], several of these organelles were subject to distintegration at the same time (Fig. 13). In these cells degradation of the peroxisomes was not always initiated by the formation of the dark membranes and then the characteristic structure of the "liquified" peroxisomes, which was so apparent

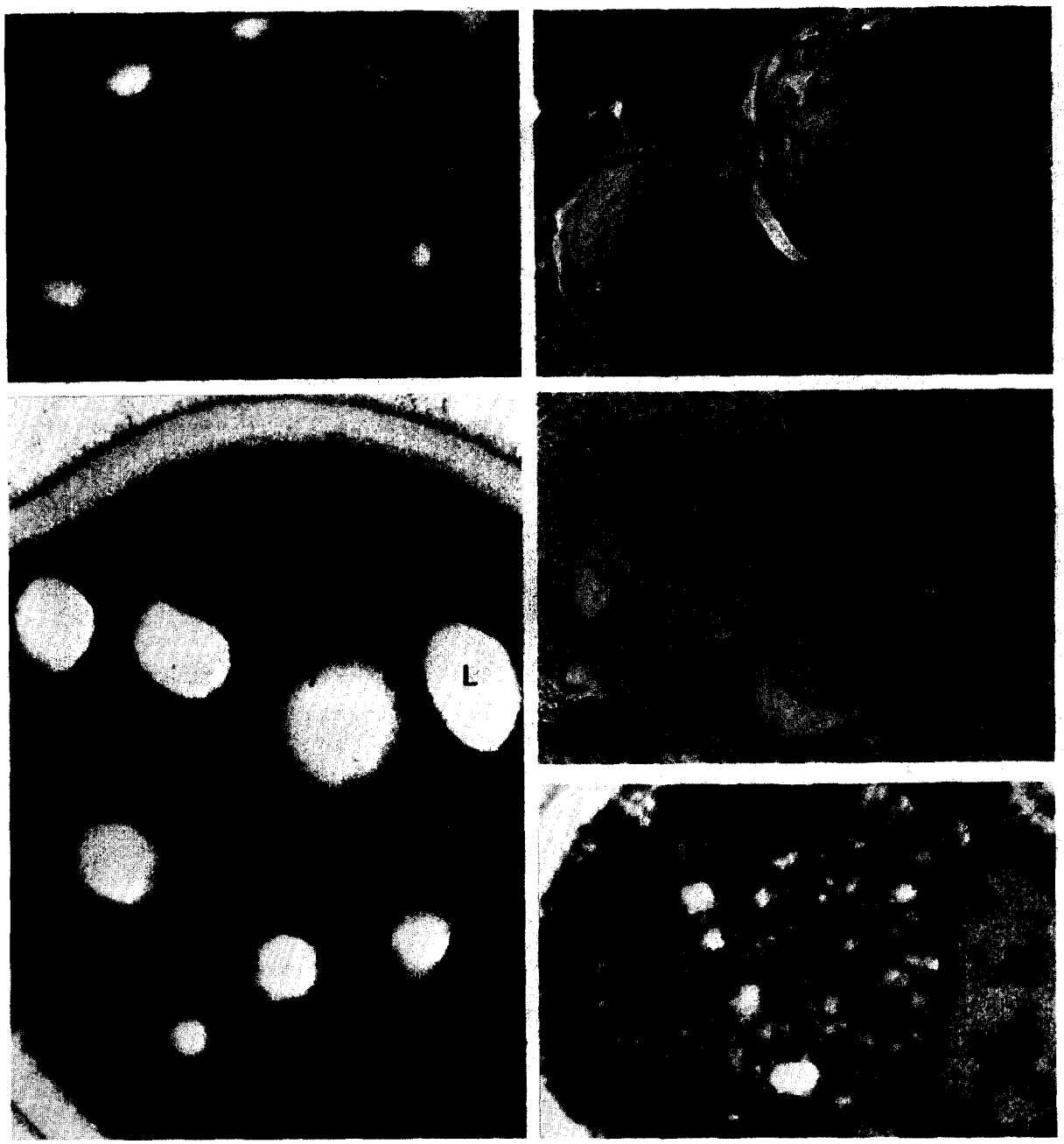


Fig. 7. Detail showing first marks of disintegration of the peroxisome (30 min after the addition of glucose). Note the change in width of the peroxisomal membrane (arrows) (61 000 ×).

Fig. 8. Freeze-fractured cell showing the membrane structure of an intact peroxisome (left) and a disintegrating peroxisome (upper right) (50 000 ×).

Fig. 9. Progressing disintegration of the peroxisomal matrix. The surrounding membrane of the organelle clearly differs from the membrane of the intact peroxisome (arrow) (54 000 ×).

Fig. 10. Detail of a freeze-fractured cell showing a membraneous invagination in the peroxisome (56 000 ×; compare Fig. 9).

Figs. 11, 12. Different stages of progressing breakdown of the peroxisomes (40 000 ×).

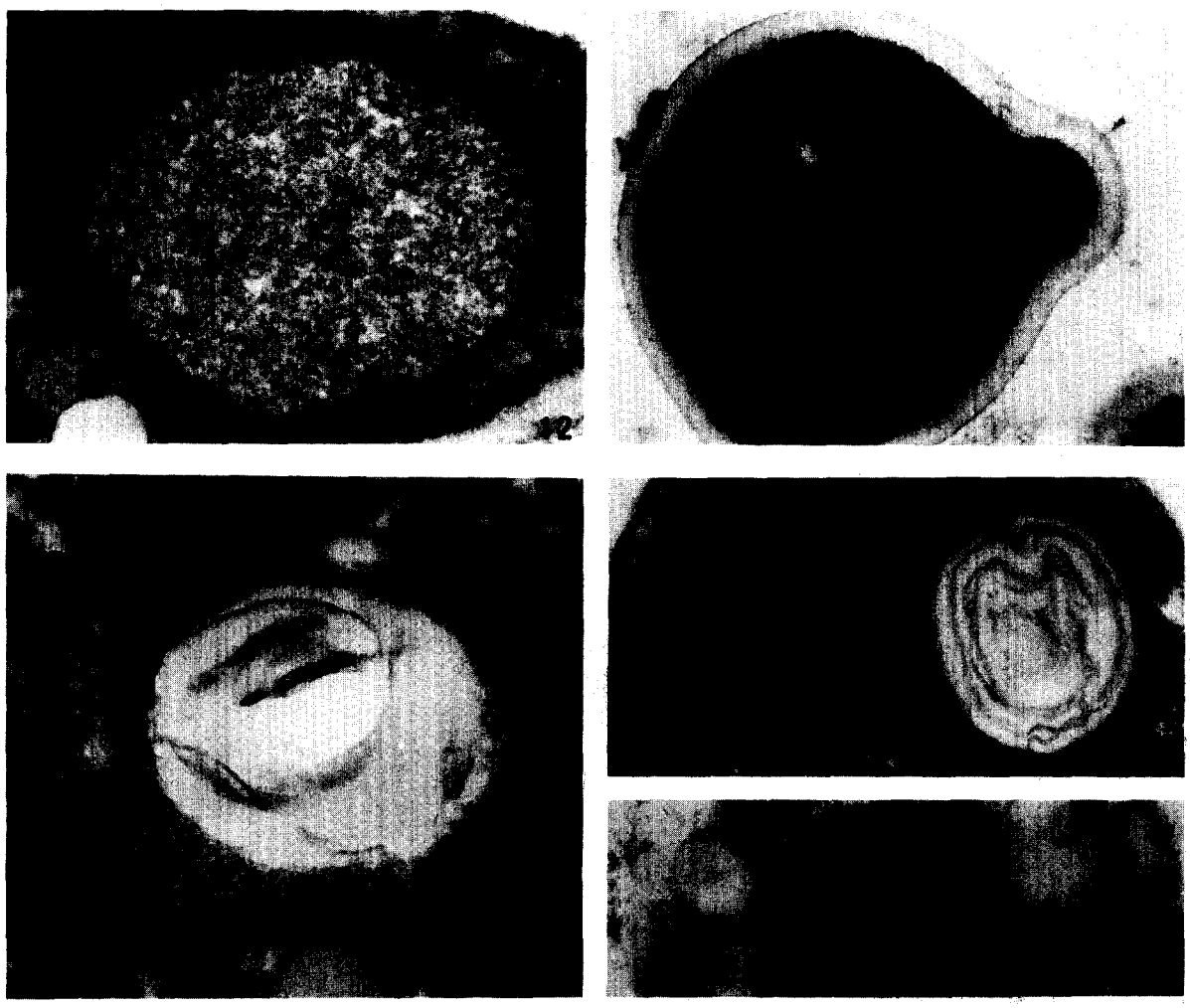


Fig. 13. Stationary phase cell, 30 min after the addition of glucose. Several peroxisomes show marks of disintegration (31 000 ×).

Figs. 14–16. Details of glutaraldehyde prefixed cells, after cytochemical staining for catalase or methanol oxidase activity and post-fixation with OsO_4. Fig. 14 shows a detail of a disintegrating peroxisome after incubation with DAB and H_2O_2. The centre of disintegration shows membraneous structures; the matrix is not stained (63 000 ×). The unaffected peroxisomes showed catalase activity (Fig. 15; 45 000 ×) and methanol oxidase activity (Fig. 16; 63 000 ×).

in exponentially growing cells (Fig. 6) were not observed.

Disintegration of peroxisomes did not start at the same time in all cells. In a number of cells the first indications of breakdown became visible within 30 min after glucose had been added to the culture, but cells in which peroxisomal degradation started after approximately 2 h of incubation in the glucose medium were also observed. However, once started, the process of peroxisomal disintegration can be completed within 60 min. The areas of peroxisomal degradation, which appeared as "holes" after $KMnO_4$-fixation (Figs. 7, 9, 13), were found to contain electron-dense deposits or membraneous structures

after fixation with glutaraldehyde and OsO_4 (Fig. 14). Since freeze-etch replicas also showed that these areas cannot be considered empty, it is concluded that the occurrence of these "holes" in Figs. 7, 9 and 13 is due to an artefact caused by the fixative.

Incubation of methanol-grown cells in glucose-containing media did not lead to the disappearance of all peroxisomes in these cells. Especially the small peroxisomes sometimes remained intact. These intact peroxisomes had a crystalline matrix and cytochemical staining techniques revealed the presence of alcohol oxidase and catalase activity in these organelles (Figs. 15, 16). These observations are in agreement with the biochemical results, which showed that part of the enzyme activities remained after 3 h. In order to establish the extent of the peroxisomal degradation, the average number and volume density of the organelles remaining after 4 h of incubation in the glucose medium were determined using morphometrical techniques. The data (Table 1) indicate a drastic decrease in both number and volume density of the peroxisomes. Since the volume density decreased twice as fast as the number of peroxisomes, the data also support the finding that especially the large peroxisomes are susceptible to early degradation.

The molecular mechanisms underlying the observed changes in the ultrastructure of methanol-grown *Hansenula polymorpha* upon transfer into glucose-containing media, and the decrease in activities of two enzymes involved in the metabolism of methanol associated with it, are unknown. Attempts to demonstrate any increase in proteolytic activity in cells in which rapid degradation of peroxisomes had been initiated [20], have so far failed. It became, however, clear that once the crystalline matrix of the peroxisome was destroyed (Fig. 6), the demonstration of activity of methanol oxidase or catalase by cytochemical means was no longer possible. Whether this inactivation, which also was apparent in cell-free extracts (Figs. 1, 2), and the subsequent development of areas of disintegration which appeared in the peroxisomal matrix (Figs. 7, 9) are due to proteolysis, remains to be seen.

TABLE 1

Effect of addition of 0.4% glucose on average number and volume of peroxisomes in cells of *Hansenula polymorpha* grown in batch cultures on methanol and taken from the mid-exponential or stationary growth phase. The number of peroxisomes is expressed as the average number per section, the volume as percentage of the cytoplasmic volume.

	Exponentially growing cells (A_{663} = 2.2)		Stationary phase cells (A_{663} = 3.4)	
	Number of peroxisomes	Volume of peroxisomes	Number of peroxisomes	Volume of peroxisomes
t = 0	1.0	20.0%	1.3	28.1%
t = 4	0.2	1.9%	0.2	2.2%

Acknowledgement

We wish to thank J. Zagers for skillful assistance in preparing the figures and the electron micrographs.

References

[1] van Dijken, J.P., Veenhuis, M., Kreger-van Rij, N.J.W. and Harder, W. (1975) Arch. Microbiol. 102, 41–44.
[2] Fukui, S., Tanaka, A., Kawamoto, S., Yasuhara, S., Terashini, Y. and Osumi, M. (1975) J. Bacteriol. 123, 317–328.
[3] Sahm, H., Roggenkamp, R. and Wagner, F. (1975) J. Gen. Microbiol. 88, 218–222.
[4] Hazeu, W., Batenburg-van den Vegte, W.H. and Nieuwdorp, P.J. (1975) Experientia (Basel) 31, 926–927.
[5] Roggenkamp, R., Sahm, H., Hinkelman, W. and Wagner, F. (1975) Eur. J. Biochem. 59, 231–236.
[6] Veenhuis, M., van Dijken, J.P. and Harder, W. (1976) Arch. Microbiol. 111, 123–135.
[7] van Dijken, J.P., Veenhuis, M., Vermeulen, C.A. and Harder, W. (1975) Arch. Microbiol. 105, 261–267.
[8] Fukui, S., Kawamoto, S., Yasuhara, S., Tanaka, A., Osumi, M. and Imaizumi, F. (1975) Eur. J. Biochem. 59, 261–266.
[9] Tanaka, A., Yasuhara, S., Kawamoto, S. and Fukui, S. (1976) J. Bacteriol. 126, 919–927.
[10] Roggenkamp, R., Sahm, H. and Wagner, F. (1974) FEBS Lett. 41, 283–286.
[11] Yasuhara, S., Kawamoto, S., Tanaka, A., Osumi, M. and Fukui, S. (1976) Agr. Biol. Chem. 40, 1771–1780.
[12] Sahm, H. and Wagner, F. (1973) Arch. Mikrobiol. 90, 263–268.
[13] Kitamura, K., Kaneda, T. and Yamamoto, Y. (1971) Arch. Biochem. Biophys. 145, 402–404.

[14] Osumi, M., Fukuzumi, F., Terashini, Y., Tanaka, A. and Fukui, S. (1975) Arch. Microbiol. 103, 1–11.
[15] van Dijken, J.P., Otto, R. and Harder, W. (1976) Arch. Microbiol. 111, 137–144.
[16] Lück, H. (1963) in: Methods of Enzymatic Analysis (H.U. Bergmeyer, Ed.), pp. 885–894, Academic Press, New York.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[18] Moor, H. (1964) Z. Zellforsch. 62, 546–580.
[19] Weibel, E.R. and Bolender, P. (1976) in: Principles and Techniques of Electron Microscopy (M.A. Hayat, Ed.), van Nostrand Reinhold, London.
[20] Holzer, H. (1976) Trends in Biochem. Sci. 1, 178–181.